In re Patent Application of: Takashi Tanaka et al.

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For: PRODUCTION OF PHYSIOLOGICALLY ACTIVE PROTEINS USING GENE RECOMBINANT SILKWORMS

TRANSLATOR'S DECLARATION

Honorable Commissioner of Patents & Trademarks
Washington, D.C. 20231

Sir:

I, Tsumoru Fukumoto , residing at c/o SEIWA PATENT & LAW, Toranomon 37 Mori Bldg., 3-5-1, Toranomon Minato-ku, Tokyo 105-8423, Japan declare the following:

(1) That I know well both the Japanese and English languages;

(2) That I translated Japanese Patent Application No. 2002-268726 , filed September 13, 2002 , from the Japanese language to the English language;

(3) That the attached English translation is a true and correct translation of the aforesaid Japanese Patent Application No. 2002-268726 to the best of my knowledge and belief; and

(4) That all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such false statements may jeopardize the validity of the application or any patent issuing thereon.

March 23, 2007
Date

Translator
Tsumoru Fukumoto

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[DESTINATION] To Commissioner, Patent Office
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[TITLE OF THE INVENTION] Vector for Gene Transfer Into Insects and Method for Manufacture of Gene Products

[NUMBER OF CLAIMS] 21

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[NAME OF DOCUMENT] SPECIFICATION

[TITLE OF THE INVENTION] Vector for Gene Transfer Into Insects and Method for Manufacture of Gene Products

[SCOPE OF CLAIM FOR PATENT]

[CLAIM 1]

A gene cassette for expression of an exogenous protein, comprising

(1) a promoter for expression in silk glands and

(2) a gene comprising the 5'-terminal portion of the fibroin H chain gene fused to the 5'-end of the exogenous protein structural gene, linked downstream from the promoter of (1) above.

[CLAIM 2]

A gene cassette for expression of an exogenous protein, comprising

(1) a promoter for expression in silk glands and

(2) a gene comprising the 3'-terminal portion of the fibroin H chain gene fused to the 3'-end of the exogenous protein structural gene containing no termination codon, linked downstream from the promoter of (1) above.

[CLAIM 3]

A gene cassette for expression of an exogenous protein, comprising

(1) a promoter for expression in silk glands and

(2) a gene comprising the 5'-terminal portion of the fibroin H chain gene fused to the 5'-end of the exogenous protein structural gene containing no termination codon, and the 3'-terminal portion of the fibroin H chain gene fused to the 3'-end of said structural gene, linked downstream from the promoter of (1) above.

[CLAIM 4]

A gene cassette according to claim 1 or 3, characterized in that the 5'-terminal portion of said fibroin H chain gene includes the first exon, the first intron and a portion of the second exon of the fibroin H chain gene.

[CLAIM 5]

A gene cassette according to claim 4, characterized in that the portion where the first exon and second exon of said fibroin H chain gene are joined is the secretory signal sequence region of the fibroin H chain gene.

[CLAIM 6]

A gene cassette according to claim 5, characterized in that (1) the promoter for expression in silk glands and (2) the 5'-terminal portion of the fibroin H chain gene linked downstream from the promoter of (1) above consist of DNA represented by SEQ ID NO: 1 or SEQ ID NO: 2.

[CLAIM 7]

A gene cassette according to claim 2 or 3, characterized in that the 3'-terminal portion of said fibroin H chain gene comprises at least one codon coding for cysteine.

[CLAIM 8]

A gene cassette according to claim 7, characterized in that the 3'-terminal portion of said fibroin H chain gene consists of DNA represented by SEQ ID NO: 3.

[CLAIM 9]

A gene cassette according to any one of claims 1 to 8, characterized in that said promoter for expression in silk glands is at least one promoter selected from among fibroin H chain gene promoter, fibroin L chain gene promoter and serine gene promoter.

[CLAIM 10]

A gene cassette according to any one of claims 1 to 9, characterized in that at least one polyA added region selected from among fibroin H chain gene polyA added region, fibroin L chain gene polyA added region and sericin gene polyA added region is present downstream from a cassette for expression of an exogenous protein according to any one of claims 1 to 9.

[CLAIM 11]

A gene cassette for gene transfer into insect cell chromosomes, characterized in that a pair of piggyBac

transposon inverted repeats are present at both ends of a gene cassette for expression of an exogenous protein according to any one of claims 1 to 10.

[CLAIM 12]

An expression vector for insect cells, characterized by comprising a gene cassette for expression of an exogenous protein according to any one of claims 1 to 11.

[CLAIM 13]

A gene transfer vector for insect cells, characterized by comprising a cassette for gene transfer into insect chromosomes according to claim 12.

[CLAIM 14]

A method for production of an exogenous protein, characterized by transferring a vector for insect cells according to claim 12 or 13 into insect cells.

[CLAIM 15]

A method for production of an exogenous protein according to claim 14, characterized in that the insect cells are derived from a *Lepidoptera* insect.

[CLAIM 16]

A method for production of an exogenous protein according to claim 15, characterized in that the insect cells are derived from *Bombyx mori*.

[CLAIM 17]

A method for production of an exogenous protein according to claim 16, characterized in that the insect cells are silk gland cells of *Bombyx mori*.

[CLAIM 18]

A method for production of an exogenous protein characterized by utilizing the DNA transposition activity of a gene transfer vector for insect cells according to claim 13 and piggyBac transposase to create a recombinant silkworm having incorporated into its chromosomes a gene cassette for expression of an exogenous protein according to any one of claims 1 to 10, producing the exogenous protein in the silk

glands or silk of the obtained recombinant silkworm, and then recovering the exogenous protein from the silk glands or silk.

[CLAIM 19]

A method for production of an exogenous protein according to claim 18, characterized in that the gene transfer vector for insect cells and the piggyBac transposase are simultaneously microinjected into silkworm eggs to create a recombinant silkworm having incorporated into its chromosomes a gene cassette for expression of an exogenous protein.

[CLAIM 20]

A recombinant silkworm having transferred into its chromosomes a gene cassette for expression of an exogenous protein according to any one of claims 1 to 10, and having the ability to produce the exogenous protein in the silk glands or silk.

[CLAIM 21]

Silk comprising an exogenous protein produced by a recombinant silkworm according to claim 20.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[Technical Field of the Invention]

The present invention relates to a vector for gene transfer into insect cells and to a method for exogenous protein production using insect cells, insect tissue or insects in which a gene has been transferred using the vector. The invention further relates to silk containing exogenous proteins produced by recombinant silkworms.

[0002]

[Prior Art]

Production of exogenous proteins utilizing gene recombinant technology has become commonly utilized in a variety of industries. The host cells used are mainly *E. coli*, yeast, animal cells, plant cells, insect cells or the like. However, no host has been developed which can efficiently

produce different types of exogenous proteins, such that is necessary to construct a production system for each protein of interest, and therefore a further technological revolution has been desired for production of exogenous proteins in individual hosts.

[0003]

Post-translational modification has been a problem in *E. coli* or other bacterial or yeast systems, and sometimes an adequately functional protein cannot be synthesized. Animal cells generally synthesize proteins in functional form, but growth is usually difficult and productivity is too low to be economical.

[0004]

On the other hand, it is known that production of gene recombinant proteins using insects or insect cells allows enzymes or useful proteins with physiological activity to be produced at high volume and relatively low cost, and that post-translational modification of the proteins is similar to that in mammals. Specifically, high-volume production of recombinant proteins at relatively low cost is possible by a method of infecting insects or insect cells with *Baculovirus* incorporating exogenous protein genes, and physiologically active proteins marketed as pharmaceuticals are known (Japanese Unexamined Patent Publication SHO No. 61-9288, Japanese Unexamined Patent Publication SHO No. 61-9297).

[0005]

However, since the conventional techniques for production of recombinant proteins with insects or insect cells use recombinant viruses for transfer of the exogenous genes, they must be inactivated or contained for safety reasons. In methods of inoculating silkworms with recombinant viruses, the procedure of viral inoculation is complicated and the recombinant proteins of interest are produced in body fluids, and must therefore be purified from the large volumes of contaminating proteins deriving from silkworm body fluids.

This has hampered efforts to obtain highly pure recombinant proteins.

[0006]

On the other hand, recombination of exogenous genes into insect chromosomes has been attempted in recent years, and specifically a method of using DNA from *Autographa californica* nuclear polyhedrosis virus (AcNPV) to transfer a fused gene, comprising the silkworm fibroin L chain gene with the jellyfish green fluorescent protein gene, into a silkworm chromosome by homologous recombination and expressing the fused gene has been developed (Genes Dev., 13 511-516, 1999); a silkworm having the human collagen gene transferred therein and a method for its production have also been developed utilizing the aforementioned technique (Japanese Unexamined Patent Publication No. 2001-161214). Recently, a method of stably transferring an exogenous gene into silkworm chromosome using the *Lepidoptera*-derived transposon piggyBac and expressing the protein encoded by the exogenous gene has been researched using jellyfish green fluorescent protein as a model, and it has been confirmed that the gene is stably passed to offspring by breeding (Nature Biotechnology 18, 81-84, 2000).

[0007]

However, since the method of transferring an exogenous protein gene into insect chromosomes using AcNPV employs a recombinant *Baculovirus* (AcNPV), the problem of inactivation or containment of the recombinant virus remains. In cases which use the piggyBac transposon, the green fluorescent protein is not produced in sufficiently large amounts, while its production throughout the entire silkworm body means that sophisticated purification techniques must be used to recover the expressed recombinant green fluorescent protein in a highly purified form, thus presenting an economical obstacle.

[0008]

In other words, such techniques for production of exogenous proteins using insect cells as hosts are associated

with several problems including the required inactivation or containment of the recombinant *Baculovirus*, and difficulty in purifying the target proteins from silkworm fluids which contain large amounts of contaminating proteins.

[0009]

[Problems to be Solved by the Invention]

Thus, while techniques for production of recombinant proteins using insects have been avidly researched, they have required containment of recombinant *Baculovirus* incorporating the exogenous protein genes, while inoculation with recombinant viruses is also a complicated procedure. In addition, the production of exogenous proteins in silkworms using recombinant *Baculovirus* has been hampered by the problem that extraction and purification of target proteins from body fluids containing large amounts of impurities is difficult.

[0010]

Although research has been carried out on techniques for production of recombinant proteins by transfer of exogenous protein genes into silkworm chromosomes, the target exogenous protein productivity has been low, and it has been difficult to purify the target proteins from silkworm body fluids.

The present invention has been accomplished in light of these circumstances, and its object is to provide a gene engineering material for insects which allows a protein of interest to be easily purified without the need to use recombinant *Baculovirus*, as well as a method for production of an exogenous protein utilizing the gene engineering material.

[0011]

[Means for Solving the Problems]

The present inventors focused on producing large volumes of fibroin, which makes up 70-80% of silk protein, and secreting it outside of silk gland cells, and as a result of diligent investigation found that a drastic improvement can be achieved in the production volume of an exogenous protein by transferring into silk gland cells a gene cassette comprising

the 5'-terminal end of the exogenous protein gene linked continuously within the amino acid frame to the 3'-terminal end of the 5'-terminal portion of the fibroin H chain gene including the first intron, downstream from a promoter for expression in silk glands.

[0012]

It was also found that when a fused gene comprising the 3'-terminal portion of the fibroin H chain gene linked continuously within the amino acid frame to the 3'-end of an exogenous protein gene is expressed under the control of a promoter for expression in silk glands, the exogenous protein is secreted in large volume outside of the silk gland cells. Furthermore, it was found that by constructing a gene cassette designed with a DNA sequence consisting of the 5'-terminal portion of the fibroin H chain gene including the first intron at the 5' end of the exogenous protein gene and a DNA sequence consisting of the 3'-terminal portion of the fibroin H chain gene at the 3'-end of the exogenous protein gene, both continuously within the amino acid frame, and creating a recombinant silkworm having the gene cassette transferred into its chromosomes, the recombinant silkworm produces the target protein in large volume in its silk.

[0013]

In other words, the present inventors have succeeded in producing large volumes of an exogenous protein in silk gland cells, outside of silk gland cells and in silk, by transferring into silk gland cells or the like an expression gene cassette comprising a DNA sequence consisting of the 5'-terminal portion and a DNA sequence consisting of the 3'-terminal portion of the fibroin H chain gene fused to the exogenous protein gene, and succeeded in establishing a technique for production of exogenous proteins which facilitates purification, by utilizing silk glands to produce the exogenous protein without requiring recombinant *Baculovirus*.

The present invention relates to the gene cassette

described below, to a gene engineering material such as a vector which can be used for production of an exogenous protein in insects, to transformants, to a method for production of the exogenous protein utilizing the transformants, and to silk comprising the exogenous protein.

[0014]

1) A gene cassette for expression of an exogenous protein, comprising

(1) a promoter for expression in silk glands and

(2) a gene comprising the 5'-terminal portion of the fibroin H chain gene fused to the 5'-end of the exogenous protein structural gene, linked downstream from the promoter of (1) above.

2) A gene cassette for expression of an exogenous protein, comprising

(1) a promoter for expression in silk glands and

(2) a gene comprising the 3'-terminal portion of the fibroin H chain gene fused to the 3'-end of the exogenous protein structural gene containing no termination codon, linked downstream from the promoter of (1) above.

[0015]

3) A gene cassette for expression of an exogenous protein, comprising

(1) a promoter for expression in silk glands and

(2) a gene comprising the 5'-terminal portion of the fibroin H chain gene fused to the 5'-end of the exogenous protein structural gene containing no termination codon, and the 3'-terminal portion of the fibroin H chain gene fused to the 3'-end of the structural gene, linked downstream from the promoter of (1) above.

4) An expression vector for insect cells, characterized by comprising a gene cassette for expression of an exogenous protein according to any one of 1) to 3) above.

5) A method for production of an exogenous protein, characterized by transferring a vector for insect cells

according to 4) above into insect cells.

[0016]

6) A method for production of an exogenous protein, characterized by creating a recombinant silkworm having incorporated into its chromosomes a gene cassette for expression of an exogenous protein according to any one of 1) to 3) above, producing the exogenous protein in the silk glands or silk of the obtained recombinant silkworm, and then recovering the exogenous protein from the silk glands or silk.

7) A recombinant silkworm having transferred into its chromosomes a gene cassette for expression of an exogenous protein according to any one of 1) to 3) above, and having the ability to produce the exogenous protein in the silk glands or silk.

8) Silk comprising an exogenous protein produced by a silkworm according to 7) above.

[0017]

[Preferred Mode of the Invention]

A "gene cassette for expression of an exogenous protein" according to the invention means a DNA set necessary for expression of a protein encoded by the exogenous protein structural gene when transferred into insect cells. The exogenous protein expression cassette comprises the exogenous protein structural gene and a promoter which promotes expression of the gene. Ordinarily, it will also comprise a terminator and polyA added region, and preferably it comprises a promoter, the exogenous protein structural gene, a terminator and a polyA added region. In addition, a secretory signal sequence may be linked between the exogenous protein structural gene and the promoter. An optional gene sequence may also be linked between the structural gene and the polyA added sequence. An artificially designed and synthesized gene sequence may also be linked.

[0018]

A "gene cassette for gene transfer" is a gene cassette for

expression of an exogenous protein having a pair of piggyBac transposon inverted repeats at both ends, and it is a DNA set which is transferred into insect cell chromosomes by the action of piggyBac transposase.

[0019]

There are no particular restrictions on the method of obtaining the DNA used for the invention. There may be mentioned a method of amplification of the necessary gene region using the PCR (polymerase chain reaction), based on known genetic information, and a method of screening from a genome library or cDNA library according to homology based on known genetic information. According to the invention, the genes include genetic polymorphisms and mutant forms produced by artificial mutagenic treatment using mutagenic agents. A genetic polymorphism is a partial variation of the base sequence of a gene due to spontaneous mutation of the gene.

[0020]

The promoter in the exogenous protein expression cassette is not particularly restricted, but is preferably one with high activity for promoting expression of exogenous protein genes. As examples there may be mentioned *Drosophila* heat shock protein promoter described in Japanese Unexamined Patent Publication HEI No. 6-261770 or Japanese Unexamined Patent Publication SHO No. 62-285787 and silkworm actin gene promoter (Nature Biotechnology 18, 81-84, 2000), and preferably there may be mentioned promoters having high promoting activity in silkworm silk gland cells, such as fibroin H chain gene promoter (nucleotide bases 255-574 of GenBank Accession No. V00094), fibroin L chain gene promoter (Gene, 100:151-158: GenBank Accession No. M76430) and sericin gene promoter (nucleotide bases 599-1656 of GenBank Accession No. AB007831).

[0021]

The "exogenous protein structural gene" is a gene not found in the host cells to be used for expression of the gene and coding for a protein which is not naturally produced by the

host cells, but it is not otherwise restricted. From the standpoint of industrial value, there may be mentioned genes for proteins produced by humans or other mammals, such as growth hormones, cytokines, growth factors and cytoskeleton proteins. Genes for enzymes or other proteins produced by microorganisms, plants or insects are also included within the scope of the invention.

[0022]

The 5'-terminal portion of the fibroin H chain gene included in the gene cassette for expression of an exogenous protein according to the invention is a DNA sequence having the function of reinforcing promoter-mediated expression of the exogenous protein gene, and it is a DNA sequence comprising the full length or part of the first exon and first intron and part of the second exon of the fibroin H chain gene.

[0023]

Production volume of the exogenous protein can be increased by fusing the 5'-end of the exogenous protein structural gene to the 3'-end of the second exon with continuity of the amino acid frame. However, if the second exon portion is too long, excess amino acid residues will be added to the N-terminal end of the desired exogenous protein, which may result in loss of the structure and activity of the desired exogenous protein, and therefore it will be necessary to adjust the length as appropriate depending on the purpose. In most cases, the second exon portion may be linked immediately after or up to a few amino acid residues from the secretory signal sequence of the fibroin H chain gene in order to achieve satisfactory results.

[0024]

The 3'-terminal portion of the fibroin H chain gene is a DNA sequence having the effect of secreting the exogenous protein in large volume outside of silk gland cells when the exogenous protein is produced in the silkworm silk glands. The recombinant silkworm has transferred into its chromosomes the

exogenous protein expression gene cassette having fused at the 3'-end thereof the 3'-terminal portion of the fibroin H chain gene as the secretory signal in silk, and it is capable of producing the exogenous protein in its silk.

[0025]

At least one cysteine residue is present in this portion, and when the 3'-terminal of the fibroin H chain gene is utilized directly, the cysteine residue is at the 20th position from the carboxyl terminal of the fibroin H chain protein. This cysteine performs the role of binding with the fibroin L chain by a disulfide bond. The length of the DNA sequence of the 3'-terminal portion of the fibroin H chain gene is not particularly restricted so long as it does not inhibit formation of the disulfide bond with the fibroin L chain. The fibroin H chain has a repeating DNA sequence which continues for more than about 100 nucleotides upstream from the 3'-terminal, and therefore it is difficult to cut and modify the DNA sequence of the upstream portion to a desired length with restriction endonucleases.

[0026]

In order to facilitate genetic engineering, therefore, the fibroin H chain 3'-terminal portion is preferably approximately 100 base pairs at the 3'-end after the end of the repeating DNA sequence of the fibroin H chain gene. If the 3'-terminal portion of the fibroin H chain gene is long, more carboxyl terminal amino acids of the fibroin H chain protein will be bonded at the carboxyl terminal of the exogenous protein, potentially impairing the structure or activity of the desired exogenous protein. Thus, depending on the desired protein, the DNA sequence of the 3'-terminal portion of the fibroin H chain gene may need to be as short as possible.

[0027]

There are no particular restrictions on the polyA region, and the polyA region of a protein gene expressed in large volume in silk glands, such as fibroin H chain, fibroin L chain

or sericin may be suitably used.

The vector of the invention is one having a circular DNA structure or a linear DNA structure. A vector which is replicable in *E. coli* and has a circular DNA structure is particularly preferred. The vector may also incorporate a marker gene such as an antibiotic resistance gene or a jellyfish-derived green fluorescent protein gene, in order to facilitate selection of transformants.

[0028]

There are no particular restrictions on the insect cells used for the invention, but they are preferably cells derived from a *Lepidoptera* insect, more preferably *Bombyx mori*, and even more preferably silkworm silk gland cells or *Bombyx mori* egg cells. Silk gland cells carry out abundant synthesis of fibroin protein, and particularly silkworm fifth-instar larva posterior silk gland cells are easily manipulated and are preferred.

[0029]

There are no particular restrictions on the method of transferring the exogenous protein expression gene cassette and vector into the insect cells. As methods for transfer into cultured insect cells there may be used the calcium phosphate method, electroporation methods, liposome methods, gene gun methods and microinjection methods, and for transfer into silkworm silk gland cells, for example, the gene may be conveniently transferred by using a gene gun on posterior silk gland tissue extracted from the body of a silkworm fifth-instar larva.

[0030]

Gene transfer into the posterior silk gland with a gene gun may be accomplished, for example, by using a particle gun by BioRad (Model PDS-1000/He) to spray gold particles coated with a vector carrying the gene cassette for expression of the exogenous protein onto posterior silk glands fixed onto an agar plate or the like, at a He gas pressure of 1100-1800 psi.

Microinjection may be appropriately used for transfer of the gene into cells contained in *Bombyx mori* eggs. For microinjection into eggs, it is not necessary to accomplish direct microinjection into cells of the eggs, but rather the eggs may simply be microinjected for gene transfer.

[0031]

By microinjection of a vector carrying the "gene cassette for gene transfer" according to the invention into *Bombyx mori* eggs, it is possible to obtain recombinant silkworms having the "gene cassette for expression of an exogenous protein" of the invention transferred into its chromosomes. Following the method of Tamura et al. (Nature Biotechnology 18, 81-84, 2000), a vector carrying the "gene cassette for gene transfer" and a plasmid having the piggyBac transposase gene positioned under the control of silkworm actin promoter may be simultaneously microinjected into *Bombyx mori* eggs, and the hatched larvae raised to adults (G0), which are cross-mated within the group to obtain a second generation (G1) of silkworm larvae.

[0032]

Recombinant silkworms normally appear in the G1 generation at a frequency of 1-2%. Selection of the recombinant silkworms may be accomplished by removing the DNA from a portion of the G1 generation silkworm tissue and using primers designed based on the exogenous protein gene for PCR. Alternatively, if the gene coding for green fluorescent protein linked downstream from a promoter capable of expression in silkworm cells is introduced into the "gene cassette for gene transfer", selection of the recombinant silkworms can be easily accomplished by selecting individuals emitting green fluorescence under ultraviolet rays among the G1 generation silkworms, such as the first-instar larvae.

[0033]

Recombinant silkworms may also be obtained in the same manner as above, by microinjecting the vector carrying the "gene cassette for gene transfer" into *Bombyx mori* eggs

simultaneously with microinjection of the piggyBac transposase protein, in order to obtain recombinant silkworms having the "gene cassette for expression of an exogenous protein" transferred into the chromosomes.

[0034]

The piggyBac transposon is a transposable element of DNA having a 13-bp inverted repeat at both ends and an ORF of approximately 2.1 kbp between them. The piggyBac transposon used for the invention is not particularly restricted, and for example, it may be derived from *Trichoplusia ni* cell line Tn-368, *Autographa californica* NPV (AcNPV) or *Galleria mellonea* NPV (GmMNPV). Preferably, plasmids pHA3PIG and pPIGA3GFP (Nature Biotechnology 18, 81-84, 2000) carrying portions of *Trichoplusia ni* cell line Tn-368 piggyBac are used to prepare the gene with piggyBac transposase exhibiting DNA transfer activity.

[0035]

The gene recombinant silkworm used for the invention is a silkworm having the exogenous protein gene transferred into the silkworm chromosomes, and it is a silkworm which gives a positive signal when the silkworm chromosomal DNA is treated with a restriction endonuclease by an ordinary method and the exogenous protein labeled by an ordinary method is then used as a probe for Southern blotting. The gene locus on the chromosome in which the cytokine gene is to be transferred is not particularly restricted so long as it is a position which does not inhibit development, differentiation and growth of the silkworm. The recombinant silkworm has the ability to produce the exogenous protein in its silk gland cells, silk gland lumen and silk. The recombinant silkworm is also able to develop and mate normally, stably retain the transferred exogenous protein gene, and pass it on to offspring.

[0036]

By thus producing successive generations of recombinant silkworms to increase their number, it is possible to easily

scale up production of the exogenous protein. Mating with wild silkworms can further increase production of the exogenous protein. In such cases, it is necessary to carry out subculturing while appropriately selecting silkworms having the desired exogenous protein gene transferred therein. Here, DNA from cells obtained from any desired tissue may be used for analysis of the marker gene used for selection of the recombinant silkworms or of the presence or structure of the exogenous protein gene by PCR, Southern blotting or the like, to easily allow discrimination of offspring inheriting the recombinant silkworm gene.

[0037]

The insect cells or silkworm silk glands into which the gene cassette for expression of an exogenous protein according to the invention has been transferred may be cultured in culture medium suitable for the type of cell to produce the exogenous protein in the culture supernatant or in the cells. For example, BmN cells as silkworm ovary cells having a gene cassette for expression according to the invention transferred therein will produce the desired exogenous protein by 3 to 4 days of culturing at 27°C in TC-100 medium (PharMingen). For example, the silkworm posterior silk glands are aseptically extracted from fifth-instar larvae and then cultured in Grace's insect medium at 25°C to produce the exogenous protein. For protein production in silk glands, it is preferred to maintain a high dissolved oxygen concentration in the medium, and to conduct the culturing while performing ultrafiltration or the like to remove factors which inhibit synthesis of low molecular weight proteins which accumulate in the medium, in order to allow extended protein synthesis.

[0038]

A desired exogenous protein can be produced in large volume in the culture supernatant of silk glands having transferred therein the exogenous protein gene to which the 3'-terminal portion of the fibroin H chain gene has been fused

according to the invention. Since the contaminating protein in the silk gland culture supernatant consists almost entirely of fibroin, the desired protein can be easily purified from the silk gland culture supernatant, and as a result, the desired protein can be obtained at high purity.

The recombinant silkworm obtained according to the invention may be raised in the same manner as ordinary silkworms, and under ordinary conditions, to produce the exogenous protein. The production volume of the exogenous protein can be increased by optimizing the culturing temperature, humidity and feeding conditions particularly during the fifth-instar, depending on the desired exogenous protein.

[0039]

The recombinant silkworm having transferred therein an exogenous protein gene to which the 3'-terminal portion of the fibroin H chain gene has been fused according to the invention can also produce the desired exogenous protein at high volume in its cocoon. The desired exogenous protein can be easily purified and recovered from the cocoon. Also, the silk containing the obtained exogenous protein may be utilized for various industrial purposes, either in natural form or partially modified, depending on the function of the exogenous protein which is produced.

[0040]

The exogenous protein can be obtained by using an appropriate procedure of extraction from the silk glands or cocoon filaments of recombinant silkworms obtained according to the invention. The solvent used for extraction of the exogenous protein from the silk glands or cocoon filaments is not particularly restricted, but an aqueous solvent system is preferred in most cases. The aqueous solution used for the extraction may contain appropriate solutes to accelerate extraction of the exogenous protein. As examples there may be mentioned inorganic acids such as phosphoric acid, organic

acids such as acetic acid, citric acid and malic acid, salt, urea, guanidine hydrochloride, salts such as calcium chloride, and polar organic solvents such as ethanol, methanol, acetonitrile, acetone, and the like. There are no particular restrictions on the pH of the extraction solution, and any desired pH may be used so long as the desired exogenous protein is not inactivated at that pH.

[0041]

There are no particular restrictions on the method of isolating and purifying the extracted exogenous protein, and any ordinary protein purification method may be employed. For example, purification and isolation may be accomplished by a combination of chromatography using a silica gel carrier, ion-exchange carrier, gel filtration carrier, chelating carrier, pigment-loaded carrier or the like, with ultrafiltration, gel filtration, dialysis, desalting such as salting out, and concentration, using the original function of the useful protein of interest as an indicator. Feline interferon-ω, for example, may be recovered from potential fractions obtained by homogenizing silk glands or cocoon filaments of silkworms into which the feline interferon-ω gene has been transferred, with 20 mM phosphate buffer solution (pH 7.0). The obtained extract may then be adsorbed onto, for example, Blue Sepharose carrier and washed, and then eluted with a salt-containing buffer solution to increase the purity of the feline interferon-ω.

[0042]

The cytokines produced in this manner may be used for medical purposes or for assay, diagnostic purposes or the like, similar to cytokines produced by other conventional production methods. When used, it may be in the form of a mixture with various additives. The tissue or cocoon filament of the silkworms expressing the cytokines may be used directly or processed for use as medical or clothing fibers. Also, tissue or silk of recombinant silkworms expressing enzymes may be used directly for enzyme reactions.

[0043]

[EXAMPLES]

The present invention will now be explained in greater detail through examples, with the understanding that the invention is in no way limited by these examples.

Example 1 Preparation of *Bombyx mori* genomic DNA

Three-day-old fifth-instar silkworms were dissected and the posterior silk gland tissues were removed. After washing with 1xSSC, 200 μl of DNA extraction buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl) was added. Proteinase K (final concentration: 200 μg/ml) was added, the tissue was thoroughly ground with a grinder, and then additional 350 μl of DNA extraction buffer and 60 μl of 10% SDS were added and the mixture was incubated at 50°C for 2 hours. After then adding 500 μl of Tris-HCl saturated phenol pH 8.0 and mixing for 10 minutes, centrifugation was performed at 10,000 rpm, 5 min, 4°c and the supernatant was collected.

[0044]

After adding phenol/chloroform/isoamyl alcohol (25:24:1) in an equivalent amount to the supernatant, the mixture was centrifuged. Phenol/chloroform/isoamyl alcohol was again added and the supernatant from centrifugation was collected. An equivalent amount of chloroform/isoamyl alcohol (24:1) was then added and the mixture was centrifuged, and to the resulting supernatant there was again added chloroform/isoamyl alcohol, and the supernatant from further centrifugation was recovered. To this supernatant there was added a 1/10 volume of 3 M sodium acetate (pH 5.2), and after mixing, a 2.5-fold volume of cold ethanol was added, the mixture was allowed to stand at -80°C for 30 minutes, and centrifugation at 15,000 rpm, 10 min, 4°C produced a precipitate of genomic DNA. The DNA precipitate was washed with 70% ethanol and then air dried. It was then dissolved and diluted with RNase-containing sterile water to 100 μg/ml to prepare a genomic DNA solution.

[0045]

Example 2 Preparation of genes

The gene used was obtained by creating primers for both ends based on the known sequence, and conducting PCR with an appropriate DNA source as template. A restriction enzyme cleavage site was attached to the ends of the primers for subsequent gene manipulation.

The fibroin H chain promoter (nucleotide bases 62118-62437 of GenBank Accession No. AF226688: hereinafter, "P region") was obtained by PCR using two different primers, primer 4 (SEQ ID NO: 4) and primer 5 (SEQ ID NO: 5), with *Bombyx mori* genomic DNA as template.

[0046]

The fibroin H chain promoter/fibroin H chain gene exon 1-intron 1-exon 2 region (nucleotide bases 62118-63513 of GenBank Accession No. AF226688: hereinafter, "HP region") was obtained by PCR using two different primers, primer 4 (SEQ ID NO: 4) and primer 10 (SEQ ID NO: 10), with *Bombyx mori* genomic DNA as template.

The fibroin H chain upstream promoter/fibroin H chain gene exon 1-intron 1 region (nucleotide bases 57444-62927 of GenBank Accession No. AF226688: hereinafter, "HUP region") was obtained by PCR using two different primers, primer 12 (SEQ ID NO: 12) and primer 13 (SEQ ID NO: 13), with *Bombyx mori* genomic DNA as template.

[0047]

The feline interferon-ω gene (nucleotide bases 9-593 of GenBank Accession No. S62636, hereinafter "IC region") was obtained by PCR using two different primers, primer 6 (SEQ ID NO: 6) and primer 7 (SEQ ID NO: 7), with *Baculovirus* rBNV100 coding for the feline interferon-ω gene as template. The virus rBNV100 may be constructed, for example, by excising the feline interferon-ω gene from a plasmid extracted from *E. coli* (pFeIFN1) (FERM BP-1633) and linking it to a silkworm cloning vector (T. Horiuchi et al., Agric. Biol. Chem., 51, 1573-1580,

1987) to create a recombinant plasmid, and then cotransfecting it together with silkworm polyhedrosis virus DNA into an established silkworm cell line.

[0048]

The fibroin H chain polyA signal region (nucleotide bases 79201-79995 of GenBank Accession No. AF226688: hereinafter, "A region") was obtained by PCR using two different primers, primer 8 (SEQ ID NO: 8) and primer 9 (SEQ ID NO: 9), with *Bombyx mori* genomic DNA as template.

The fibroin H chain C-terminal region gene-fibroin H chain polyA signal region (nucleotide bases 79099-79995 of GenBank Accession No. AF226688: hereinafter, "region A") was obtained by PCR using two different primers, primer 11 (SEQ ID NO: 11) and primer 9 (SEQ ID NO: 9), with *Bombyx mori* genomic DNA as template.

[0049]

The β-galactosidase (β-gal) gene was obtained by PCR using two different primers, primer 16 (SEQ ID NO: 16) and primer 17 (SEQ ID NO: 17), with pβgal-Basic vector (Clontech) as template.

The PCR was carried out according to the manufacturer's protocol, using KODplus (Toyobo Co., Ltd.). Specifically, each template was added at 100 ng for *Bombyx mori* genomic DNA and 10 ng for *Bombyx mori* posterior silk gland cDNA and pβgal-Basic vector, and the reagents were added with each primer at 50 pmol, the included 10 x PCR buffer at 10 μl, 1 mM $MgCl_2$, 0.2 mM dNTPs and 2 units of KODplus, to a total of 100 μl. Reaction was carried out in 30 cycles using a Perkin-Elmer DNA thermal cycler under DNA denaturing conditions of 94°C, 15 seconds, primer annealing conditions of 55°C, 30 seconds and extension conditions of 68°C, 60-300 seconds.

[0050]

The reaction solutions were electrophoresed with 1% agarose gel, and an ordinary procedure was used for extraction

and preparation of a DNA fragment of approximately 0.3 kbp for the P region, approximately 1.4 kbp for the HP region, approximately 5.5 kbp for the HUP region, approximately 580 bp for the IC region, approximately 0.8 bp for the A region, approximately 0.9 bp for the HA region and approximately 3.2 kbp for the β-gal gene. The DNA fragments were phosphorylated with polynucleotide kinase (Takara Shuzo), and after cleavage with HincII, were reacted overnight with dephosphorylated pUC19 vector at 16°C, using DNA Ligation Kit Ver.2 by Takara Shuzo Co., Ltd., for ligation. The vectors were then used to transform *E. coli* by an ordinary procedure, and insertion of the PCR fragments into the obtained transformants was confirmed by PCR of the resulting colonies under the same conditions described above, upon which the PCR fragment-inserted plasmids were prepared by an ordinary procedure. The plasmids were sequenced to confirm that the obtained fragments had the respective gene base sequences.

[0051]

Example 3 Construction of β-galactosidase expression plasmids

The plasmid carrying the β-gal gene prepared in Example 2 was cut with *Sal*I and *Hind*III, and the approximately 0.3 kbp fragment (P region) excised from a plasmid carrying the fibroin H chain promoter by *Sal*I and *Hind*III was inserted therein. This was cut with *Bam*HI and the approximately 0.8 kbp fragment (A region) excised from a plasmid carrying the fibroin H chain polyA signal region by *Bam*HI was inserted therein, after which the obtained plasmid carrying the β-gal gene was purified using a QIAGEN Plasmid Maxi Kit according to the manufacturer's protocol. The obtained plasmid was named pPgalA, and it was confirmed to be the target plasmid by PCR and sequencing.

[0052]

In a similar manner, the plasmid carrying the β-gal gene prepared in Example 1 was cut with *Sal*I and *Hind*III, and the

approximately 1.4 kbp fragment (HP region) cut from a plasmid carrying the fibroin H chain promoter/fibroin H chain gene exon 1-intron 1-exon 2 region by *Sal*I and *Hind*III was inserted therein. This was cut with *Bam*HI and the approximately 0.9 kbp fragment (HA region) excised from a plasmid carrying the fibroin H chain C-terminal region/fibroin H chain polyA signal region by *Bam*HI was inserted therein, after which the obtained plasmid carrying the β-gal gene was purified using a QIAGEN Plasmid Maxi Kit according to the manufacturer's protocol. The obtained plasmid was named pHPgalHA, and it was confirmed to be the target plasmid by PCR and sequencing.

[0053]

Example 4 Construction of gene transfer plasmids

Plasmid pigA3GFP (Nature Biotechnology 18, 81-84, 2000) was used as the gene transfer plasmid. Specifically, pigA3GFP is a vector obtained by removing the transposase coding region from plasmid p3E1.2 disclosed in U.S. Patent No. 6,218,185, and inserting at the section the A3 promoter (nucleotide bases 1764-2595 of GenBank Accession No. U49854), with pEGFP-N1 vector (Clontech)-derived GFP and SV40-derived polyA added sequence (nucleotide bases 659-2578 of GenBank Accession No. U55762). This vector is available from the Japan National Institute of Agrobiological Sciences. The *Xho*I site upstream from the A3 promoter was blunted and the feline interferon-ω gene expression cassette was inserted.

[0054]

The structure of the gene expression cassette for this example was: fibroin H chain promoter/feline interferon-ω/fibroin H chain poly A signal region (P/IC/A), fibroin H chain promoter/fibroin H chain gene exon 1-intron 1-exon 2 region/feline interferon-ω/fibroin H chain C-terminal region/fibroin H chain polyA signal region (HP/IC/HA), fibroin H chain upstream promoter/fibroin H chain gene exon 1-intron 1-exon 2 region/feline interferon-ω/fibroin H chain C-terminal

region/fibroin H chain polyA signal region (HUP/IC/HA), or fibroin H chain promoter/fibroin H chain gene exon 1-intron 1-exon 2 region/feline interferon-ω/fibroin H chain polyA signal region (HP/IC/A).

[0055]

The method will now be described in detail.

The P/IC/A construct was obtained by the following method. The plasmid carrying the feline interferon-ω (IC region) prepared in Example 1 was cut with *Sal*I and *Hind*III, and the approximately 0.3 kbp fragment (P region) excised from a plasmid carrying the fibroin H chain promoter by *Sal*I and *Hind*III was inserted therein. This was cut with *Bam*HI and the approximately 0.8 kbp fragment (A region) excised from a plasmid carrying the fibroin H chain polyA signal region by *Bam*HI was inserted therein. The plasmid carrying this P/IC/A was cut with *Asc*I, and the approximately 1.7 kbp excised fragment blunted with T4 DNA Polymerase by Takara Shuzo Co., Ltd. was ligated with *Xho*I-cut, blunted and dephosphorylated pigA3GFP, to obtain a transgenic construct comprising the P/IC/A gene cassette. The method is shown schematically in Figs. 1 and 2.

[0056]

The HP/IC/HA construct was obtained by the following method. The plasmid carrying the feline interferon-ω (IC region) prepared in Example 1 was cut with *Sal*I and *Hind*III, and the approximately 1.4 kbp fragment (HP region) cut from a plasmid carrying the fibroin H chain promoter/fibroin H chain gene exon 1-intron 1-exon 2 region by *Sal*I and *Hind*III was inserted therein.

[0057]

This was cut with *Bam*HI and the approximately 0.9 kbp fragment (HA region) excised from a plasmid carrying the fibroin H chain C-terminal region/fibroin H chain polyA signal region by *Bam*HI was inserted therein. The plasmid carrying this HP/IC/HA was cut with *Asc*I, and the approximately 2.9 kbp

excised fragment blunted with T4 DNA Polymerase by Takara Shuzo Co., Ltd. was ligated with *Xho*I-cut, blunted and dephosphorylated pigA3GFP, to obtain a transgenic construct comprising the HP/IC/HA gene cassette. The method is shown schematically in Figs. 3 and 4.

[0058]

The HUP/IC/HA construct was obtained by the following method. Two different primers, primer 14 (SEQ ID NO: 14) and primer 15 (SEQ ID NO: 15) were used for PCR with 1 ng of the HP/IC/HA construct as template, to obtain an approximately 2.1 kbp fibroin H chain intron 1-exon 2 region/feline interferon-ω/fibroin H chain C-terminal region/fibroin H chain polyA signal region.

[0059]

This was cut with *Xho*I and *Sph*I, and an approximately 5.5 kbp fragment (HUP region) excised from a plasmid carrying the fibroin H chain upstream promoter/fibroin H chain gene exon 1-intron 1 region by *Xho*I and *Sph*I was inserted therein. The plasmid carrying this HUP/IC/HA was cut with *Asc*I, and the approximately 7.6 kbp excised fragment blunted with T4 DNA Polymerase by Takara Shuzo Co., Ltd. was ligated with *Xho*I-cut, blunted and dephosphorylated pigA3GFP, to obtain a transgenic construct comprising the HUP/IC/HA gene cassette. The method is shown schematically in Figs. 5 and 6.

[0060]

The HP/IC/A construct was obtained by the following method. The plasmid carrying the feline interferon-ω (IC region) prepared in Example 1 was cut with *Sal*I and *Hind*III, and the approximately 1.4 kbp fragment (HP region) cut from a plasmid carrying the fibroin H chain promoter/fibroin H chain gene exon 1-intron 1-exon 2 region by *Sal*I and *Hind*III was inserted therein.

[0061]

This was cut with *Bam*HI and the approximately 0.8 kbp fragment (A region) excised from a plasmid carrying the fibroin

H chain polyA signal region by *Bam*HI was inserted therein. The plasmid carrying this HP/IC/A was cut with *Asc*I, and the approximately 2.8 kbp excised fragment blunted with T4 DNA Polymerase by Takara Shuzo Co., Ltd. was ligated with *Xho*I-cut, blunted and dephosphorylated pigA3GFP, to obtain a transgenic construct comprising the HP/IC/A gene cassette. The method is shown schematically in Figs. 7 and 8.

[0062]

The P/IC/A transgenic construct, HP/IC/HA transgenic construct, HUP/IC/HA transgenic construct and HP/IC/A transgenic construct were purified using a QIAGEN Plasmid Maxi Kit according to the manufacturer's protocol.

[0063]

Example 5 Expression of β-galactosidase in silkworm silk glands

Gold particles with a diameter of 1.6 μm were washed and sterilized with 100% ethanol and suspended in sterilized distilled water (60 mg/ml). The β-gal gene expression cassette was transferred into silkworm silk glands using a gene gun. Specifically, 50 μl (0.3 mg) of gold particles, 10 μg of expression plasmid pPgalA or pHPgalHA obtained in Example 3, 50 μl of 2.5 M calcium chloride and 20 μl of 0.1 M spermidine were combined in that order, and after standing at room temperature for 30 minutes, the mixture was centrifuged to recover the gold particles coated with pHgalC.

[0064]

The obtained gold particles were washed twice with 70% ethanol, and then dispersed in 50 μl of 100% ethanol. A 10 μl portion of the gold particle suspension was placed on a microcarrier and dried. The gene gun used was a PDS-1000/He by BioRad. Posterior silk glands extracted from three-day-old fifth-instar silkworm larvae were gently washed twice with PBS and set on a 1% agar plate, and then sprayed with the DNA-coated gold particles at a pressure of 1100 psi. After DNA

transfer, the silk glands were transferred into 20 ml of Grace's insect medium and cultured at 25°C for 2 days. After culturing, the culture supernatant and silk gland cells were recovered for confirmation of β-gal expression.

[0065]

Expression was confirmed by Western blotting. The silk gland cells were homogenized in PBS and the cell contents were extracted. The culture supernatants and cell extracts were adjusted to a total protein concentration of 1.0 mg/ml, and were used as samples for SDS-PAGE. After blotting on a membrane, an ECL Plus™ Western Blotting Kit (Amersham-Pharmacia) was used to detect β-gal protein according to the manufacturer's protocol. Specifically, the blotted membrane was subjected to blocking overnight at 4°C in blocking solution (5% skim milk, 0.1% Tween20/PBS).

[0066]

The membrane was washed twice with TPBS (0.1% Tween20/PBS) and treated at room temperature for 1 hour with anti-β-gal protein antibody (Sigma) diluted 1000-fold with TPBS. The membrane was washed twice with TPBS and then three more times for 5 minutes each with TPBS. After then diluting 10,000-fold with TPBS, it was treated with HRP-labeled anti-rabbit IgG antibody at room temperature for 1 hour. The membrane was washed twice with TPBS and then three more times for 5 minutes each with TPBS, and then an ECL Plus™ Western Blotting Detection System (Amersham-Pharmacia) detection reagent (solution A + solution B) was added. This was followed by exposure to Hyperfilm™ ECL™ and development.

[0067]

Since β-gal protein was detected only in the silk gland cells and cell supernatants having pHPgalHA transferred therein, it was demonstrated that the region outside the fibroin H chain promoter, i.e. the fibroin H chain gene exon 1-intron 1-exon 2 region, plays an important role in protein

synthesis or gene expression in the cells. Extracellular secretion was also confirmed. The results are shown in Fig. 9.

[0068]

Example 6 Creation of gene recombinant silkworms

A 0.5 mM phosphate buffer (pH 7.0)/5 mM KCl solution was prepared containing the gene transfer plasmid described in Example 4 and the piggyBac transposase protein at about 200 μg/ml DNA concentration and about 2.7 μg/ml piggyBac transposase protein concentration (molar ratio = 1:10), and 3-20 nl thereof was microinjected into 500 silkworm eggs within 4 hours after egg production.

[0069]

The larvae hatched from the silkworm eggs were raised and the obtained adults (G0) were cross-mated within the group to obtain a second generation (G1) which was observed for the fluorescence of jellyfish green fluorescent protein, to screen for silkworms having the jellyfish green fluorescent protein transferred into their chromosomes. As a result, there were obtained gene recombinant silkworms emitting fluorescence due to the action of the jellyfish green fluorescent protein.

[0070]

Example 7 Western blotting analysis of expression of recombinant protein in silk gland tissue

The posterior silk gland tissue of non-transformed silkworms and transformed silkworms (HP/IC/A transformed silkworms, HP/IC/HA transformed silkworms and HUP/IC/HA transformed silkworms) were collected, and expression of feline interferon-ω in the tissue was examined by Western blotting. The silkworm posterior silk gland cells were homogenized in 100 mM sodium phosphate buffer (pH 7.0) and after centrifugation, the supernatant was collected as a sample and an ECL Plus™ Western Blotting Kit (Amersham-Pharmacia) was used to detect feline interferon-ω according to the manufacturer's protocol. Specifically, the blotted membrane was subjected to blocking overnight at 4°C in blocking solution (5% skim milk, 0.1%

Tween20/PBS).

[0071]

The membrane was washed twice with TPBS (0.1% Tween20/PBS) and treated at room temperature for 1 hour with anti-feline interferon antibody (Sigma) diluted 1000-fold with TPBS. The membrane was washed twice with TPBS and then three more times for 5 minutes each with TPBS. After then diluting 10,000-fold with TPBS, it was treated with HRP-labeled anti-rabbit IgG antibody at room temperature for 1 hour. The membrane was washed twice with TPBS and then three more times for 5 minutes each with TPBS, and then an ECL Plus™ Western Blotting Detection System (Amersham-Pharmacia) detection reagent (solution A + solution B) was added. This was followed by exposure to Hyperfilm™ ECL™ and development.

[0072]

As a result, no signal was detected from the posterior silk gland tissues of the non-transformed silkworms and the P/IC/A construct-transformed silkworms, but a signal was detected from the silk gland tissues of the HP/IC/A construct-, HP/IC/HA construct- and HUP/IC/HA construct-transformed silkworms. The results of this experiment reconfirmed that the region outside the fibroin H chain promoter, i.e. the fibroin H chain gene exon 1-intron 1-exon 2 region, plays an important role in drastically augmenting protein synthesis or gene expression in silkworm posterior silk gland cells. The results are shown in Fig. 10.

[0073]

Example 8 Assay of recombinant protein in silk by Western blotting

Secretion of the exogenous protein (feline interferon-ω) in silk was examined next.

Cocoons from non-transformed silkworms and transformed silkworms (HP/IC/A transgenic transformed silkworms, HP/IC/HA transgenic transformed silkworms and HUP/IC/HA transgenic transformed silkworms) were measured out to 10 μg each, 4 ml of

60% LiSCN was added and each mixture was stirred and allowed to stand overnight at room temperature for dissolution of the cocoons. The solutions were each diluted 10-fold with 8 M urea/2% SDS/5% 2-mercaptoethanol to prepare samples, and then an ECL Plus™ Western Blotting Kit (Amersham-Pharmacia) was used to detect feline interferon according to the manufacturer's protocol. A molecular imager (BioRad) was used to measure the signal strength and this was compared against the signal strength of feline interferon of known concentration to determine the protein content.

[0074]

As a result, no signal was detected from the cocoons of the non-transformed silkworms and the HP/IC/A transgenic transformed silkworms, but a signal was detected from the cocoons of the HP/IC/HA transgenic and HUP/IC/HA transgenic transformed silkworms, thereby confirming secretion of the feline interferon protein into the silk. The content was approximately 0.8-2.0% with the HP/IC/HA-transformed silkworms and approximately 0.8-5.4% with the HP/IC/HA-transformed silkworms. This corresponds to 0.4-2 mg when converted to weight per silkworm.

The results of this experiment demonstrated that the fibroin H chain gene 3'-terminal portion plays an important role in secretion into silk of proteins synthesized in the posterior silk gland cells. The results are shown in Fig. 11.

[0075]

Example 9 Assay of recombinant protein in silk by ELISA

Feline interferon-ω in silk was quantitated by ELISA.

Cocoons from non-transformed silkworms and transformed silkworms (HP/IC/A transgenic transformed silkworms, HP/IC/HA transgenic transformed silkworms and HUP/IC/HA transgenic transformed silkworms) were measured out to 10 μg each, 4 ml of 60% LiSCN was added and each mixture was stirred and allowed to stand overnight at room temperature for dissolution of the cocoons. The solutions were each diluted 8-fold or 16-fold

with PBS, and applied onto a microtiter plate. Feline interferon of known concentration serially diluted with PBS was used as the standard.

As a result, no feline interferon-ω was detected in the silk of the HP/IC/A transgenic transformed silkworms, but the content was about 1.1-2.2% in the HP/IC/HA transgenic transformed silkworms and about 1.0-4.9% in the HP/IC/HA transgenic transformed silkworms.

[0076]

[Effect of the Invention]

According to the invention it is possible to produce large volumes of an exogenous protein in silk gland cells, outside of silk gland cells and in silk, by transferring into silk gland cells or the like an expression gene cassette comprising a DNA sequence consisting of the 5'-terminal portion and a DNA sequence consisting of the 3'-terminal portion of the fibroin H chain gene fused to the exogenous protein gene. The novel method establishes a technique for production of exogenous proteins which facilitates purification, by utilizing silk glands to produce the exogenous proteins without recombinant *Baculovirus*.

[0077]

[SEQUENCE LISTING]

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[0078]

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[BRIEF DESCRIPTION OF THE DRAWINGS]

Fig. 1 is an illustration of a procedure for preparing a transgenic construct comprising a P/IC/A gene cassette (part 1).

Fig. 2 is an illustration of a procedure for preparing a transgenic construct comprising a P/IC/A gene cassette (part 2).

Fig. 3 is an illustration of a procedure for preparing a transgenic construct comprising a HP-IC-HA gene cassette (part 1).

Fig. 4 is an illustration of a procedure for preparing a transgenic construct comprising a HP-IC-HA gene cassette (part 2).

Fig. 5 is an illustration of a procedure for preparing a transgenic construct comprising a HUP-IC-HA gene cassette (part 1).

Fig. 6 is an illustration of a procedure for preparing a transgenic construct comprising a HUP-IC-HA gene cassette (part 2).

Fig. 7 is an illustration of a procedure for preparing a transgenic construct comprising a HP/IC/A gene cassette (part

1).

Fig. 8 is an illustration of a procedure for preparing a transgenic construct comprising a HP/IC/A gene cassette (part 2).

Fig. 9 is an electrophoresis photograph in lieu of a drawing, showing Western blotting analysis of β-galactosidase expression in cultured silkworm silk glands. This photograph demonstrates that the fibroin H chain gene exon 1-intron 1-exon 2 region plays an important role in synthesis of the protein or expression of the gene in the cells. Extracellular secretion was also confirmed.

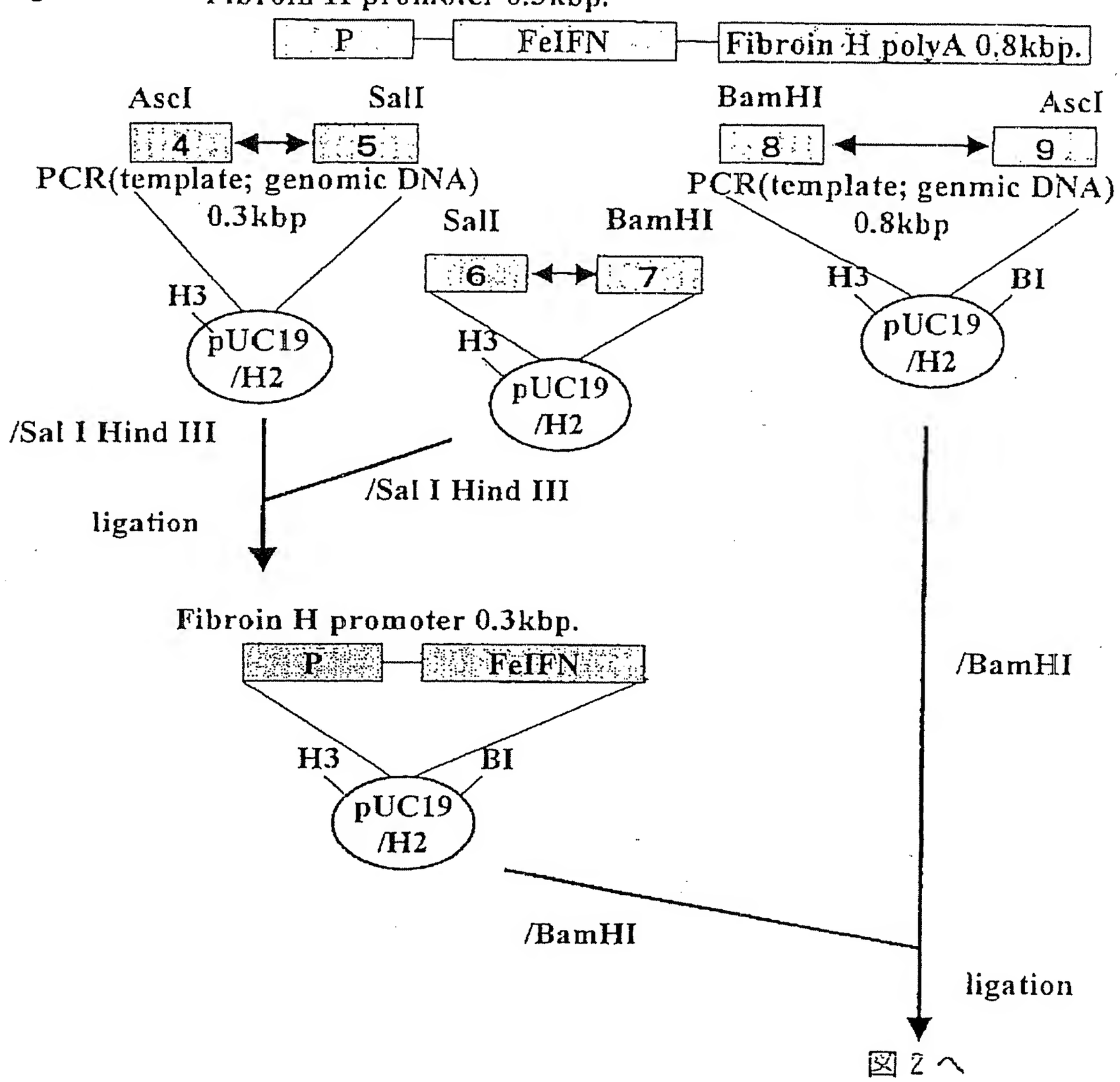
Fig. 10 is an electrophoresis photograph in lieu of a drawing, showing Western blotting analysis of recombinant protein expression in silkworm silk gland tissue. This photograph reconfirms that the fibroin H chain gene exon 1-intron 1-exon 2 region plays an important role in drastically augmenting recombinant protein expression in silkworm posterior silk gland cells.

Fig. 11 is an electrophoresis photograph in lieu of a drawing, showing Western blotting analysis of recombinant protein expression in silk. This photograph demonstrates that the fibroin H chain gene 3'-terminal portion plays an important role in secretion into silk of proteins synthesized in silk gland cells.

【書類名】　図面
【Name of Document】　Drawings

【図１】
【Fig. 1】

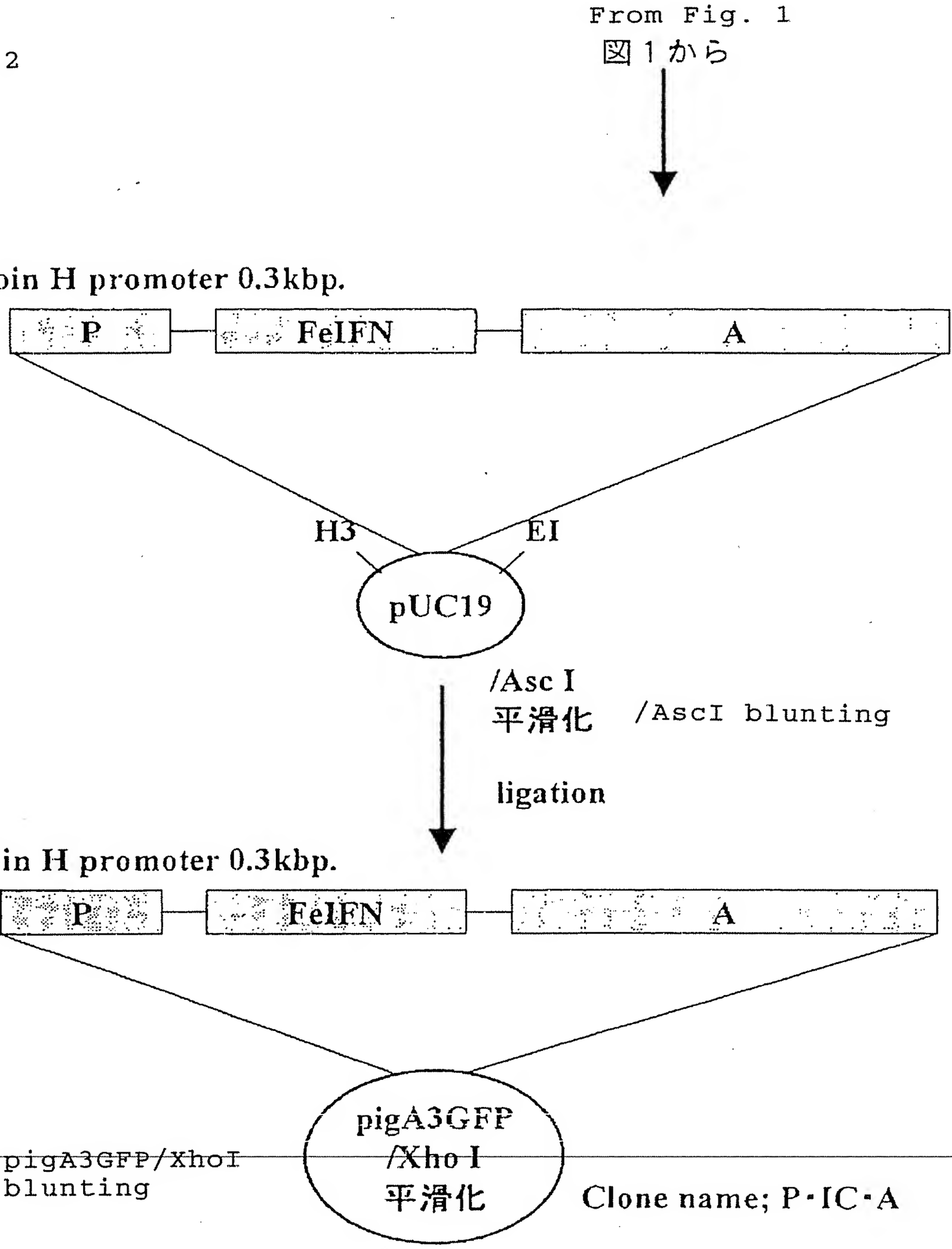
図１
Fig. 1



To Fig. 2

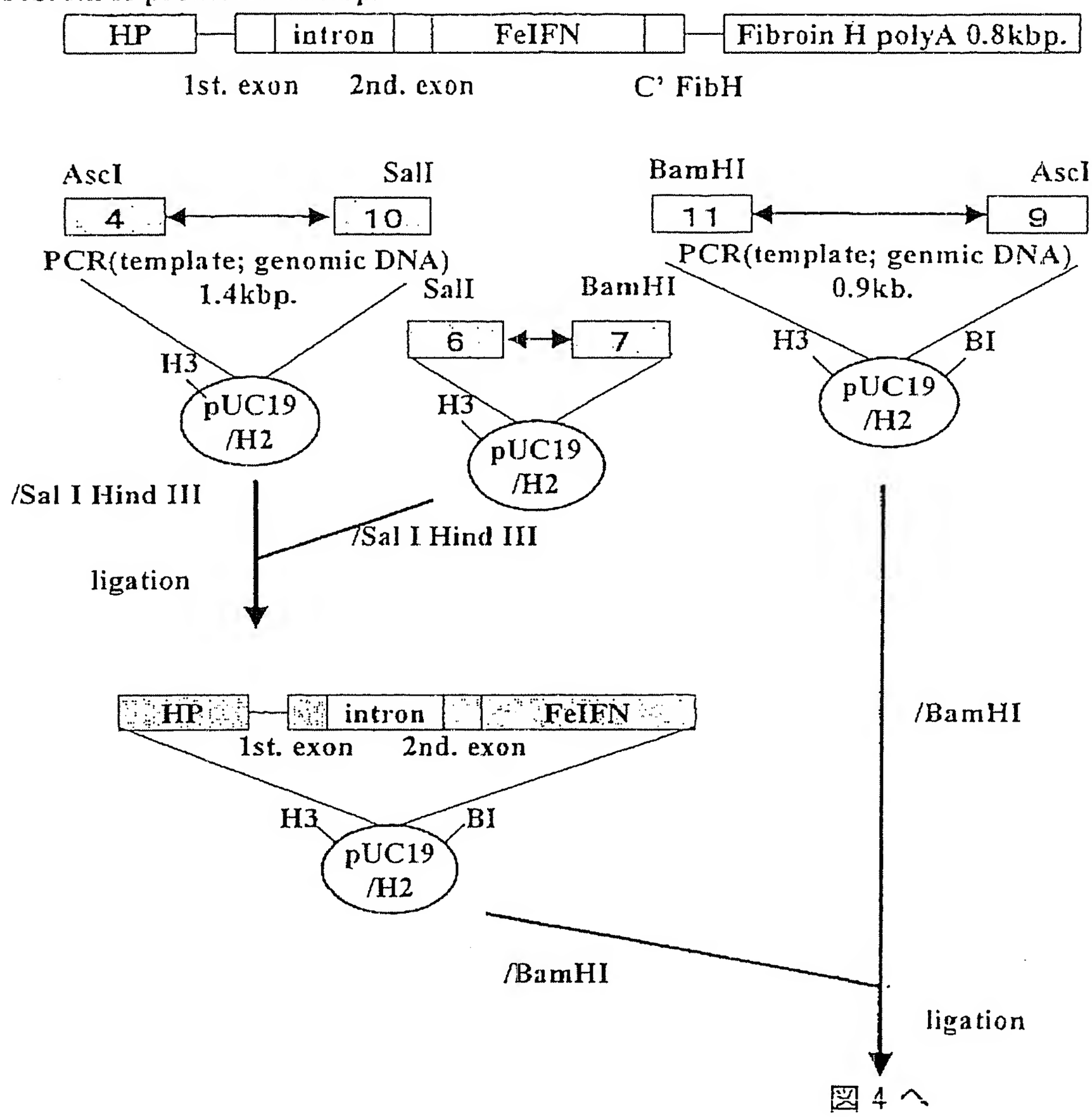
【図２】
【Fig. 2】

図２
Fig. 2



【図３】
【Fig. 3】

図３　Fig. 3



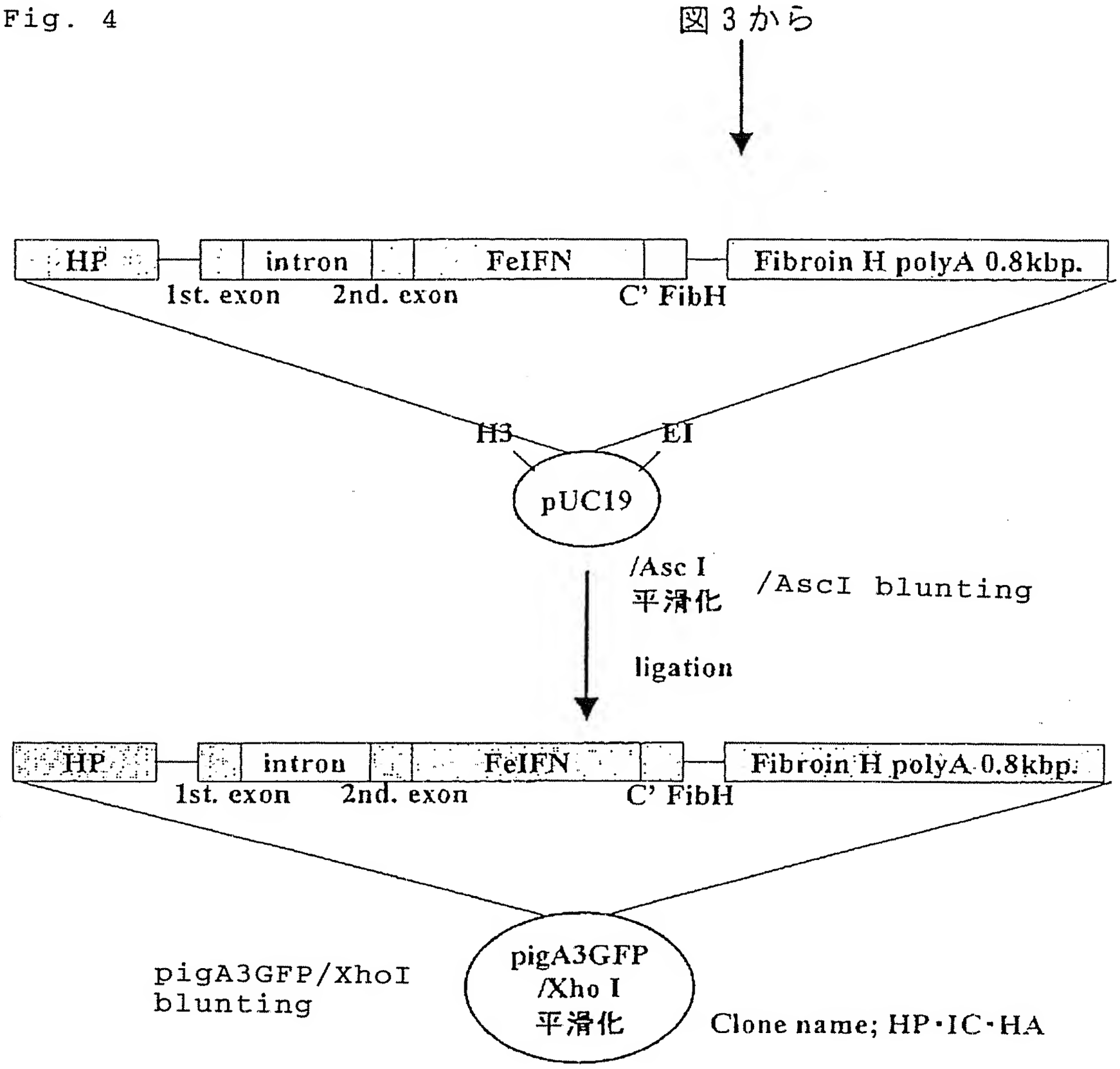
To Fig. 4

【図４】

【Fig. 4】

図 4

Fig. 4



【図５】
【Fig. 5】

図５
Fig. 5

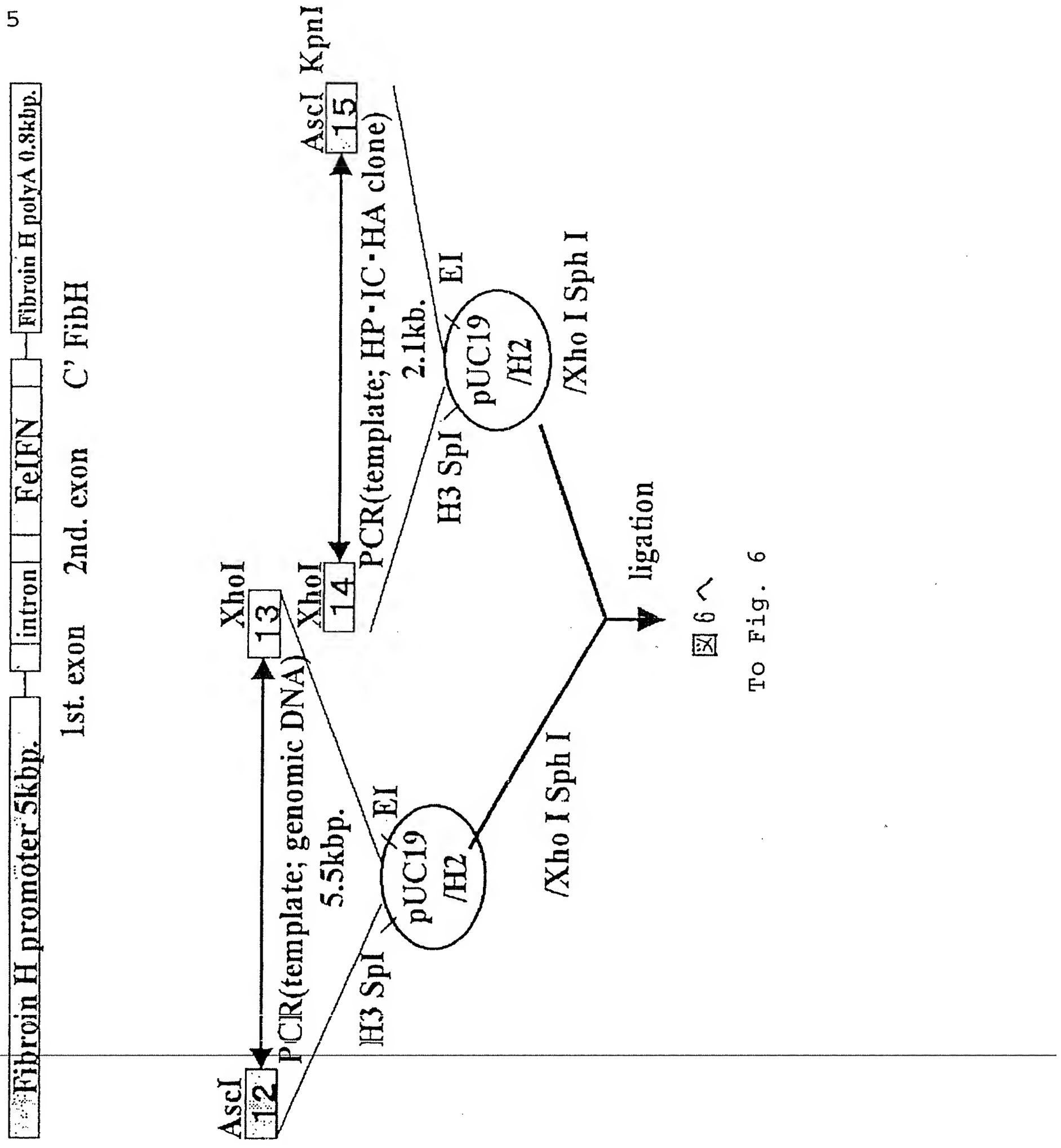
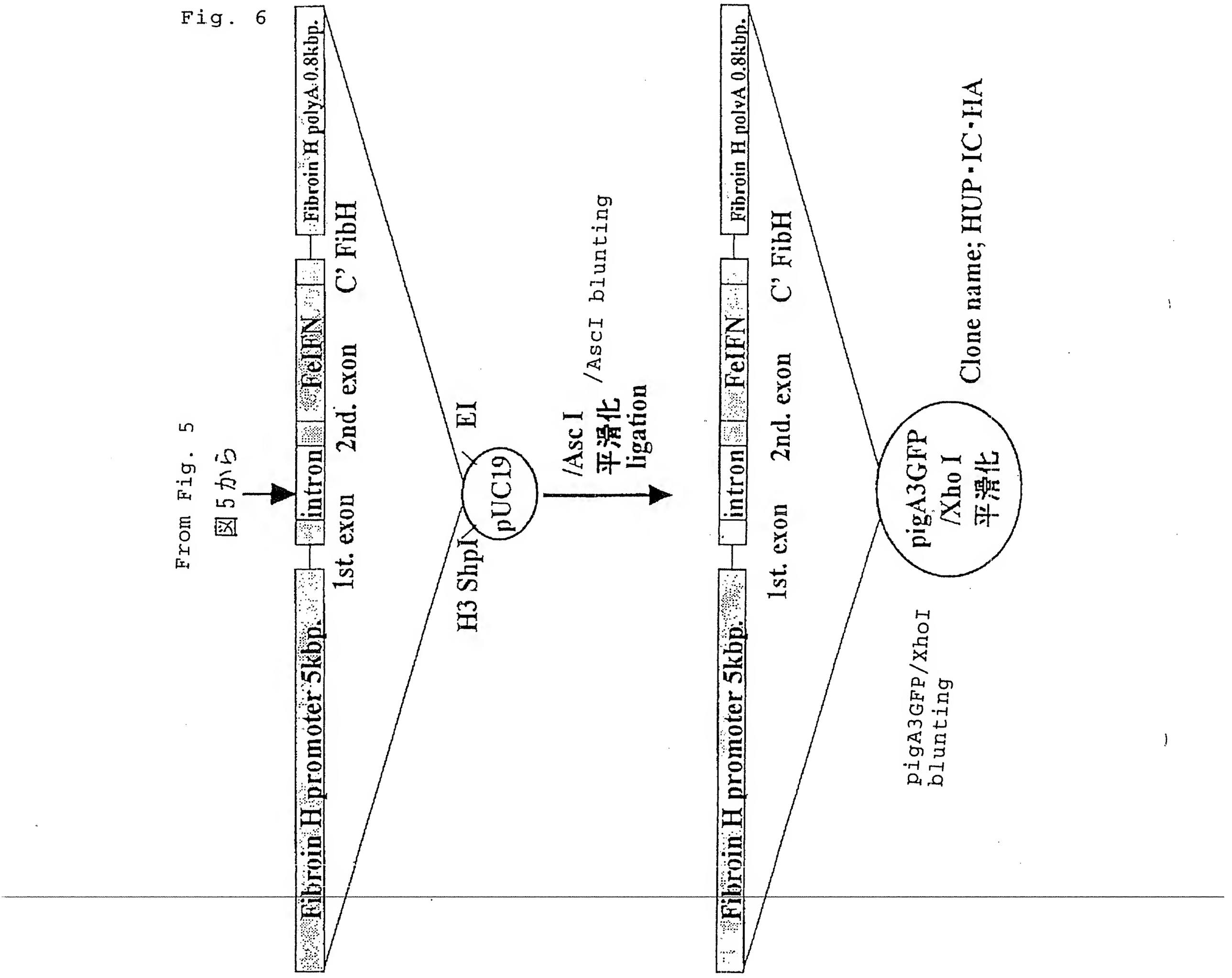


図６へ
To Fig. 6

【図６】
【Fig. 6】

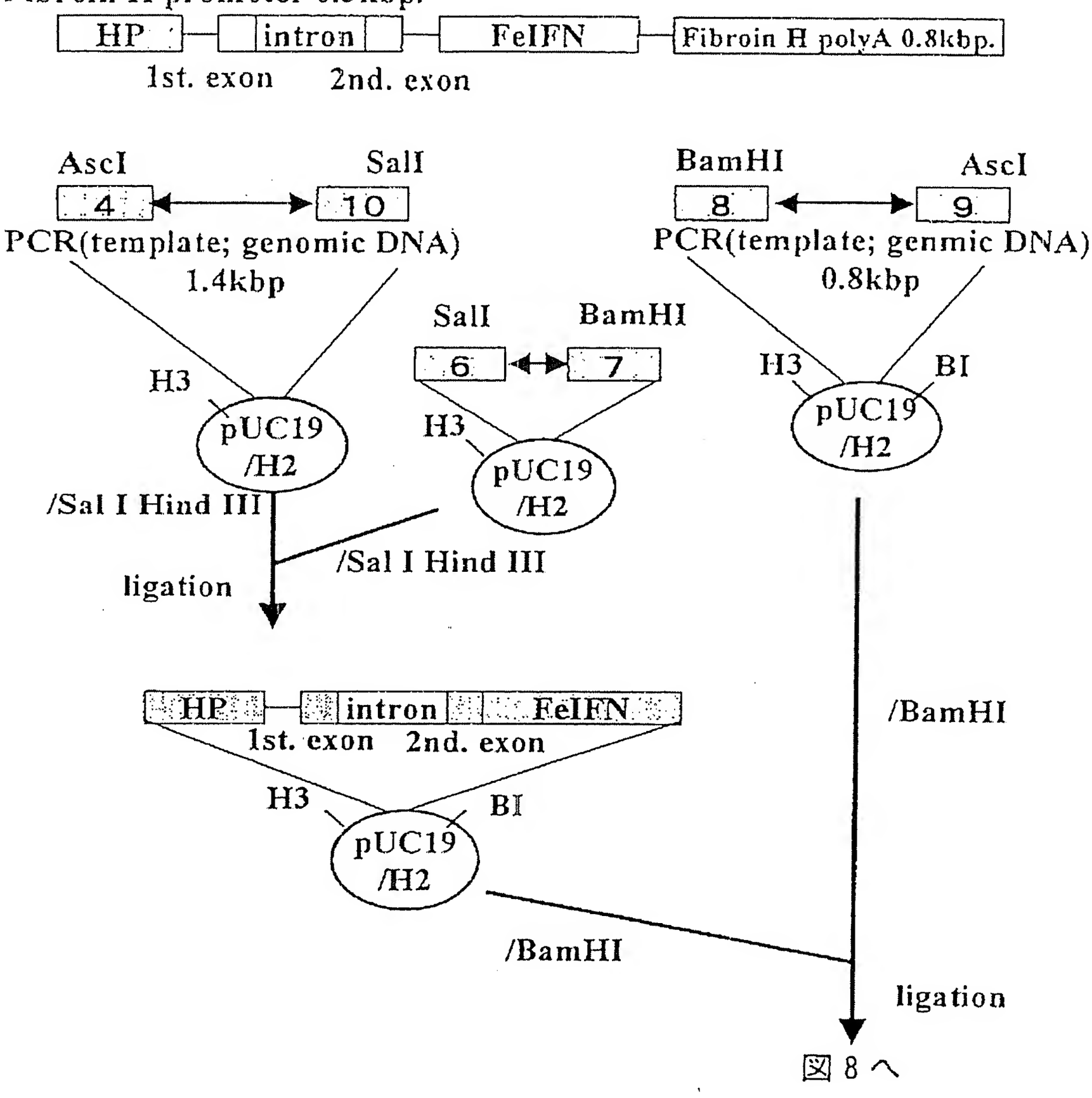
図６

Fig. 6



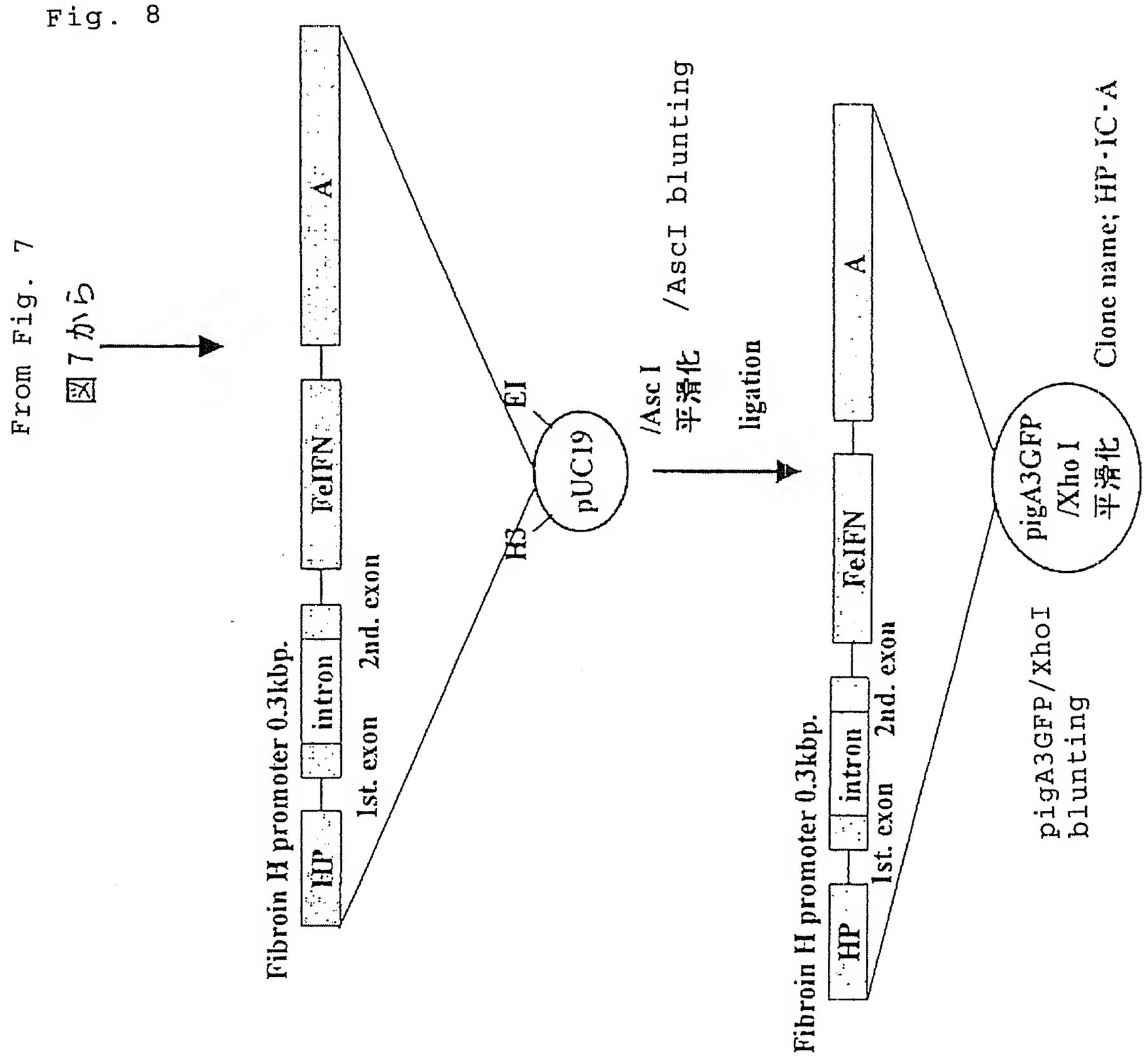
【図７】
【Fig. 7】

図 7　Fig. 7



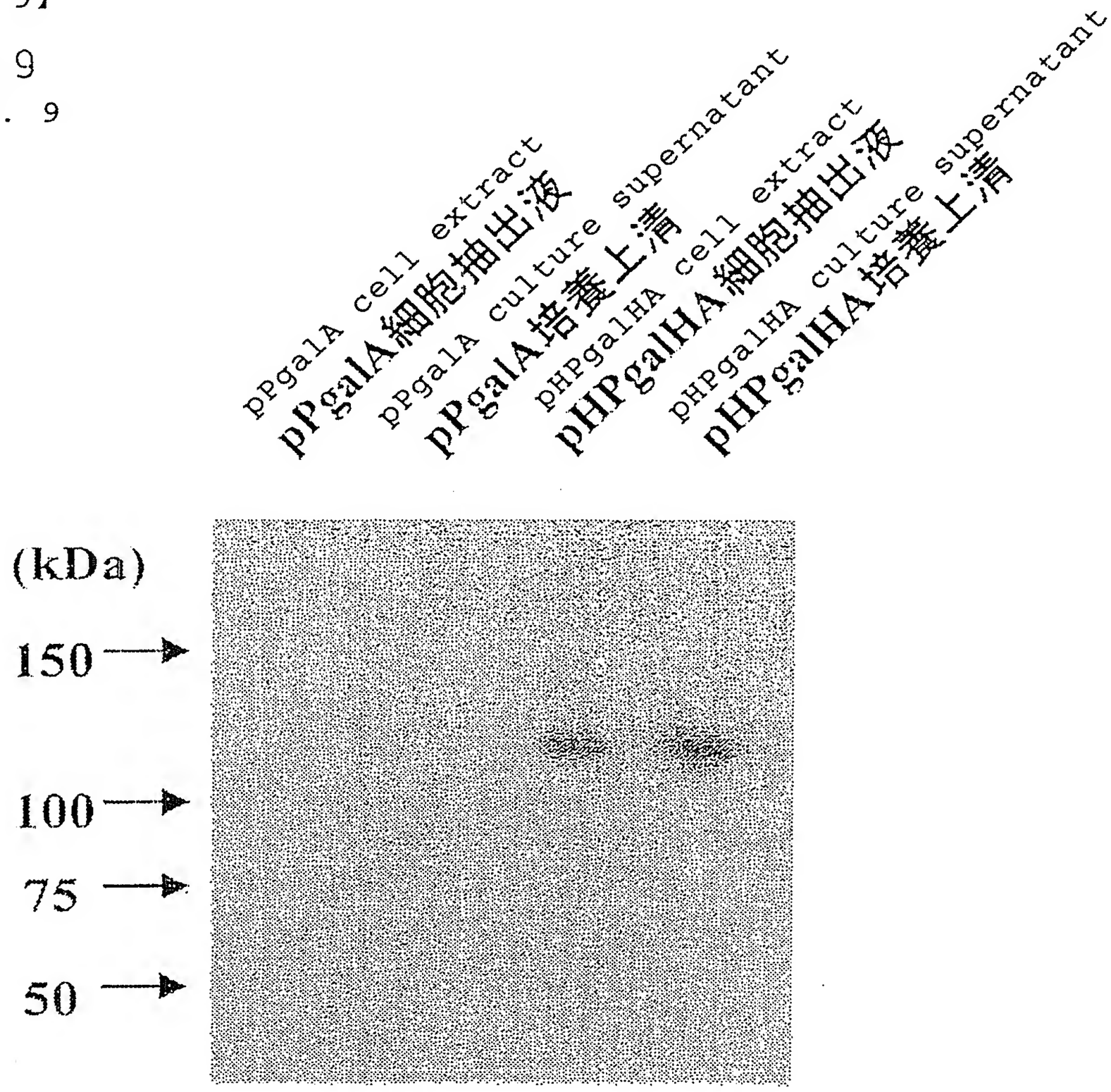
【図８】
【Fig. 8】

図８
Fig. 8



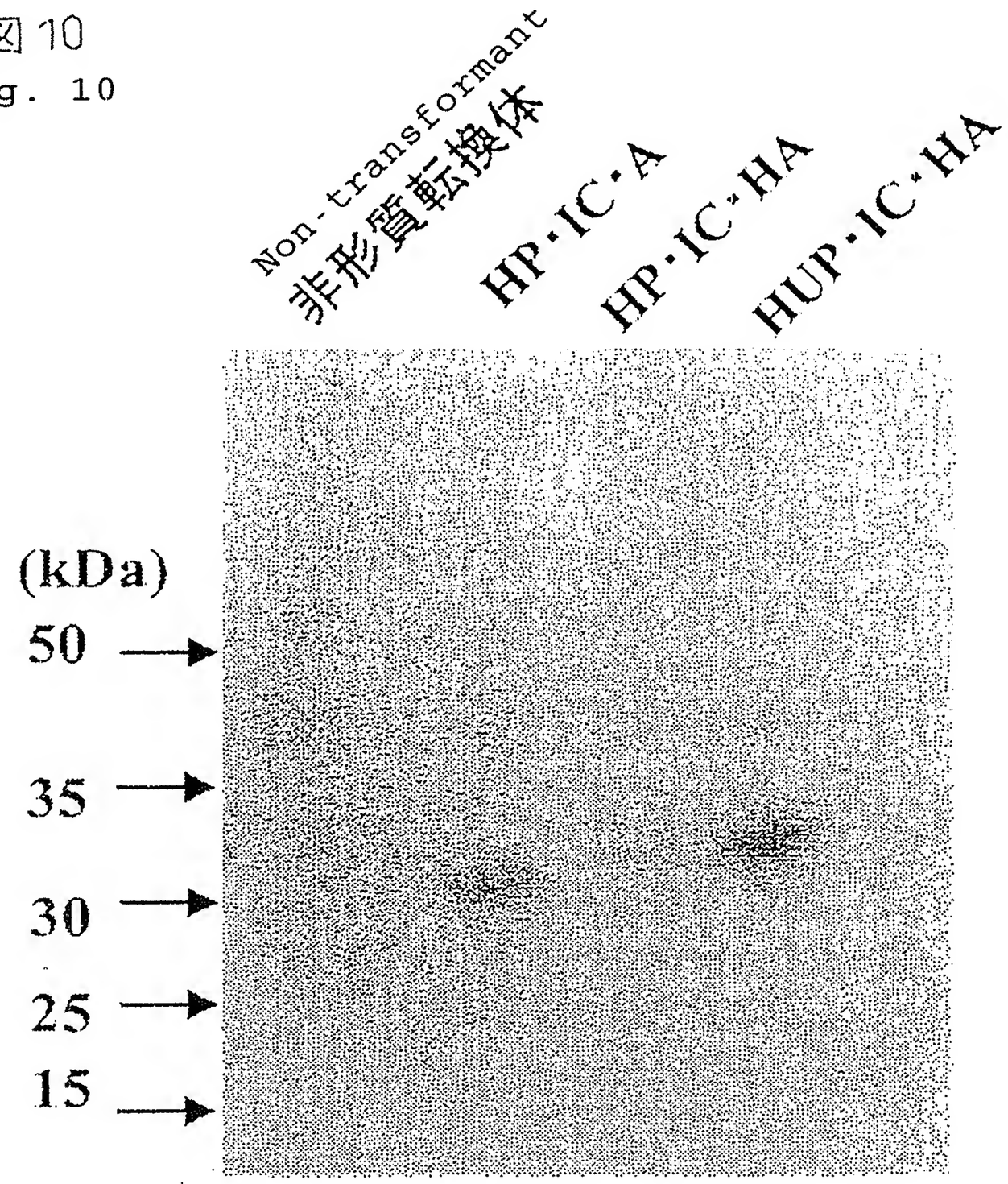
【図９】
【Fig. 9】

図９
Fig. 9



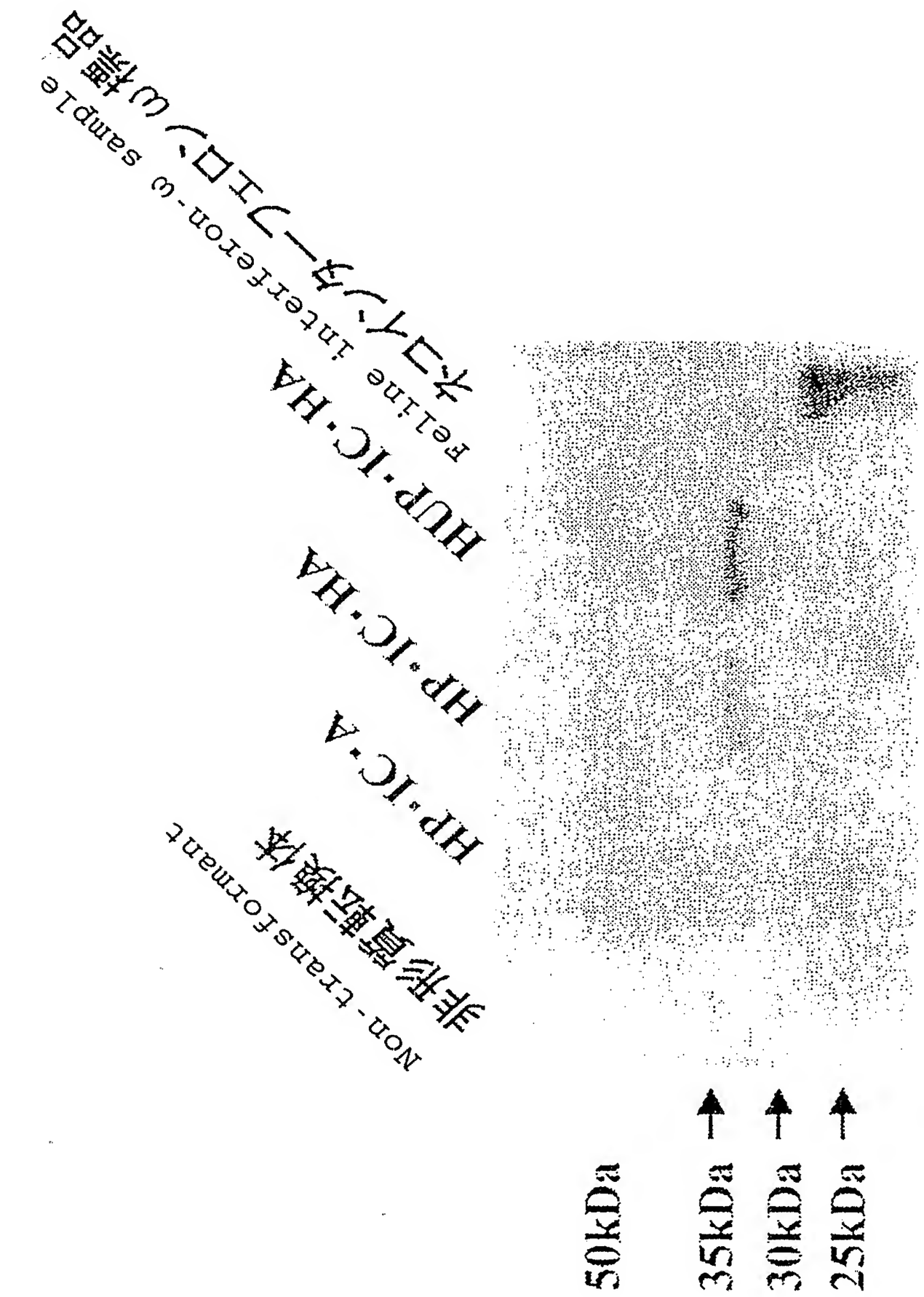
【図１０】
【Fig. 10】

図10
Fig. 10



【図１１】
【Fig. 11】

図11 Fig. 11



[NAME OF DOCUMENT] ABSTRACT

[SUMMARY]

[OBJECT]

To provide a gene engineering material for insects which allows a protein of interest to be easily purified without the need to use recombinant *Baculovirus*, as well as a method for production of an exogenous protein utilizing the gene engineering material.

[SOLUTION MEANS]

It is possible to produce large volumes of an exogenous protein in silk gland cells, outside of silk gland cells and in silk, by transferring into silk gland cells or the like an expression gene cassette comprising a DNA sequence consisting of the 5'-terminal portion and a DNA sequence consisting of the 3'-terminal portion of the fibroin H chain gene fused to the exogenous protein gene. The novel method establishes a technique for production of exogenous proteins which facilitates purification, by utilizing silk glands to produce the exogenous proteins without recombinant *Baculovirus*.

[SELECTED DRAWING] None